Protocol

Isolation of *Xenopus* Oocytes

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Xenopus oocytes and oocyte extracts are the starting material for a variety of experimental approaches. Oocytes are obtained by surgical removal of the ovary from anesthetized females. Although oocytes may be used while they remain within their ovarian follicle, it is more practical to work with defolliculated oocytes. Defolliculation can be performed either manually or enzymatically. Here we present a protocol for the isolation and separation of *Xenopus* oocytes at various developmental stages, and guidelines for maintaining oocytes in culture.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Antibiotics

Gentamycin (1000× stock solution [10 mg/mL in H₂O])

Streptomycin (1000× stock solution [100 mg/mL in H₂O])

Collagenase (Type 1; Worthington Biochemical) or Liberase TM (Roche Diagnostics) (see Step 5)

The Type 1 collagenase preparation from Worthington is a crude product known to contain collagenases I and II as well as caseinase, clostripain, and tryptic activity. Liberase TM contains highly purified collagenases I and II blended with Thermolysin, a nonclostridial neutral protease. Liberase is preferred for many applications because it is purified and has significantly higher specific activities with lot-to-lot consistency.

Medium for oocyte culture

Modified Barth's saline (MBS) for oocytes <R>

Oocyte culture medium (OCM) <R> (for long-term culture; see Step 11)

A number of formulations exist for OCM (Wallace and Misulovin 1978; Opresko et al. 1980; Wylie et al. 1996). The formulation cited here is used for host transfer studies (Schneider et al. 2010).

Oocyte reagent 2 (O-R2) <R> (Wallace et al. 1973)

The choice of medium (MBS or OR-2) depends upon what the oocytes will be used for experimentally. For longer-term culture (Step 11), OCM containing antibiotics and bovine serum albumin (BSA) is useful to maintain healthy oocytes.

Xenopus (anesthetized females)

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*The selection of the donor female will depend on the desired stage of oocytes. In *Xenopus*, oogenesis is divided into six stages, each identified by a Roman numeral (Dumont 1972). Sexually mature females will yield higher numbers of stage V–VI oocytes, whereas juvenile females may have predominantly early-stage (I–II) oocytes with few, if any, later staged oocytes. Early stages of oocytes have been ideal to image RNA localization (Heinrich and Deshler 2009; Gagnon and Mowry 2010); extracts from late-stage oocytes have been used to study the cell cycle (Novak and Tyson 1993; Iwabuchi et al. 2000) and signal transduction (Crane and Ruderman 2006).*

Equipment

Culture dishes (100-mm [glass] and 150-mm [plastic])

Dissecting microscope and dual gooseneck LED illuminators (low heat)

Forceps (Dumont #4 or #5)

For manual defolliculation (Step 3), two pairs of forceps are used: either two sharpened or one sharpened and one shaped like blunt pliers, according to preference.

Glass bowl for sequential sieving (170 mm in diameter, 90 mm high)

Incubator at 18°C

Sieves (Pro 4" or Low Pro 4") with 475- and 1000- μ m mesh (Aquaculture Nursery Farms) (see Step 9. ii)

Nylon mesh filters are also available from Cole-Parmer (U.S. Mesh 18,1000 μ m; U.S. Mesh 35,500 μ m).

METHOD

Oocyte Harvest and Defolliculation

Defolliculation can be performed manually (Step 3) or by enzymatic digestion (Steps 4–8).

1. Harvest ovarian tissue by surgery from anesthetized female frogs.
2. After surgical harvest, place the ovarian tissue in MBS and tease apart with forceps to expose the oocytes for defolliculation (Fig. 1A,A'). Examine the oocytes before defolliculation to determine the quality, and proceed only if the oocytes are healthy.

See Step 11 for a description of oocyte quality.

See Troubleshooting.

Manual Defolliculation

Manual defolliculation is recommended for experiments requiring only a few to a few hundred oocytes and for host transfer experiments (Schneider et al. 2010). Manually defolliculated oocytes can be used immediately for experiments.

Oocytes are surrounded by a vitelline membrane, follicle cells, and connective tissue rich in blood vessels (theca). Manual defolliculation is unlikely to remove the innermost layer of follicle cells. At a protein level, this cellular contamination is likely inconsequential, but may be significant at the DNA level.

3. Perform manual defolliculation using two pairs of forceps.

This procedure is well-described and shown by Schneider et al. (2010) (see <http://www.jove.com/video/1864/fertilization-of-xenopus-oocytes-using-the-host-transfer-method>) and Miyamoto et al. (2015) (see <http://www.jove.com/video/52496/manipulation-vitro-maturation-xenopus-laevis-oocytes-followed>).

Enzymatic Defolliculation

4. Cut the teased ovarian tissue into small pieces (0.5–1 cm in diameter) for enzymatic digestion.
5. Transfer ~10 mL of ovarian tissue pieces to 25 mL of enzyme solution (for a total volume of 35 mL). For enzyme solution, use either Type 1 collagenase diluted in medium to 2 mg/mL (~0.23 WU/mL; 9.2 WU/25 mL) or Liberase TM diluted in medium to ~0.26 WU/mL (0.35 mL/25 mL).

Calcium is required for optimal collagenase activity and is included in the medium.

Although enzymatic defolliculation is mainly targeting collagen fibers comprising the theca, the most effective products are not pure collagenase but a preparation that contains a mixture of proteases.

FIGURE 1. Overview of oocyte isolation. (A) A whole ovary with multiple finger-shaped lobes, each containing hundreds of oocytes, and (A') a partially opened lobe showing oocytes in follicles at various oogenic stages. (B) The sieving apparatus: a schematic depiction, and top and lateral photographic views with a 1000- μ m sieve ideal to isolate stage VI oocytes. A smaller culture dish is placed beneath the sieve within a larger glass dish, and medium (O-R2 or MBS) is added until the mesh is submerged. (C) Healthy oocytes (left to right): Stage (St.) I (50–300 μ m), a white, compact Balbiani body or mitochondrial cloud (MC) (arrow) in close contact with the nucleus (N); lateral view of late stage I with MC integrated with the vegetal cortex (arrow); Stage II (300–450 μ m), yolk deposition begins and MC (arrow) associated with vegetal cortex; Stage III (450–600 μ m) lateral view, melanogenesis begins, vegetal pole (arrow); Stages IV through VI (600–1250 μ m), uniform color, smooth “velvet” texture at the animal pole and relatively unpigmented vegetal pole, Stage VI color ranges from pale to dark brown, often displays a light equatorial band (brackets); Matured stage VI oocyte (animal pole view) showing germinal vesicle breakdown (GVBD). (D) Dying/unhealthy oocytes (left to right): Stages III through VI, dark (microbial contamination) or white spots (cellular degeneration or necrosis) evident (arrows) with uneven pigmentation; Stage I diffuse MC (arrows).

6. Digest the tissue pieces at room temperature (22°C–24°C) with rotation. Monitor digestion every 30 min to assess progress, as overtreatment with the enzyme can irreversibly damage the oocytes making them unsuitable for use.

This process typically takes 1.5–2.5 h. Digesting at a higher temperature (25°C–30°C) speeds the process, however temperatures above 24°C activate heat-shock processes. It is uncertain if oocytes fully recover from heat-shock even after overnight recovery times (Davis and King 1989).

Stage V–VI oocytes are released first by enzymatic treatment. If earlier stage oocytes are desired, the clumps of tissue can be further digested.

7. End digestion by pipetting the freed oocytes into 150-mm plastic culture dishes containing 100 mL of medium.
8. Wash oocytes twice with 100 mL of medium per wash.

Isolation of Oocyte Stages

9. Sort the different stages of oocytes by hand or using a sequential sieving process as follows.
 - i. Place a 100-mm glass culture dish beneath a sieve within a larger glass bowl (Fig. 1B). Add medium until the mesh filter of the sieving apparatus is submerged.
 - ii. Gently pipette the cells onto the submerged mesh filter.

The glass culture dish catches the oocytes that pass through the filter. Larger oocytes (Stages V–VI) are collected on a 1000-μm filter with Stage I–IV oocytes passing through. Stages III–IV are collected on a 475-μm filter with Stage I–II and some early Stage III oocytes passing through.
 - iii. Retrieve the oocytes in the culture dish by removing the buffer from the glass bowl until the culture dish can be removed without disturbing the cells.
 - iv. Transfer oocytes collected on the filter to culture dishes containing medium throughout the procedure.

Oocyte Maintenance in Culture

10. Culture defolliculated oocytes overnight in medium at 18°C to allow recovery from the effects of the enzymatic digestion.
11. Culture oocytes in medium for up to 4 d at 18°C, changing medium twice daily. Remove degrading cells with each medium change to prevent deleterious effects on the healthy cells.

The addition of antibiotics and BSA to the culture medium (as included in OCM) is useful to maintain healthy oocytes during longer-term culturing.

Overcrowding will cause the oocytes to degrade and die. Generally, a 100-mm culture dish holds thousands of stages I–II oocytes, ~1000 oocytes at stages III–IV, and up to 500 oocytes at stages V–VI.

Under a dissecting microscope with epi-illumination (Fig. 1C), healthy stage I oocytes will have a white, compact mitochondrial cloud (also called Balbiani body); stage II oocytes will have a disc/cone of mitochondrial cloud material attached to the vegetal cortex; the animal hemisphere of stages III through VI will have a smooth, velvety color; stage VI oocytes will often display a lighter band between the animal and vegetal pole. The color of the animal pole can vary from a light gray in stage III oocytes to almost black in later stages; healthy stage VI oocytes can range in color from pale to dark brown. Dying or unhealthy oocytes in stages III through VI will present dark or white spots and mottled or uneven pigmentation at the animal pole. Unhealthy stages I and II oocytes will have diffuse mitochondrial clouds and/or a deflated appearance (Fig. 1D).

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 2): Harvested oocyte quality is poor.

Solution: Inadequate frog maintenance (water quality, crowded tanks, unsuitable food) (Hilken et al. 1995) or parasitic infestation, such as flukes, are common reasons for poor oocyte quality. Proceed with a different frog.

Problem (Step 11): Oocyte degradation is observed during culture.

Solution: Solutions at the incorrect pH, extended enzymatic treatment during defolliculation, crowded culture dishes, or microbial contamination (Elsner *et al.* 2000; O'Connell *et al.* 2011) can contribute to oocyte degradation in culture. Add antibiotics to the medium, such as streptomycin or gentamycin (100 µg/mL), and check the pH. Reduce the cell density in the culture dishes. Remove degrading cells with each change of medium.

RECIPES

Modified Barth's Saline (MBS) for Oocytes

1. Prepare a 0.1 M CaCl_2 solution by dissolving 1.11 g of CaCl_2 in deionized H_2O for a final volume of 100 mL.
2. Prepare a 10× MBS salt solution by dissolving the following reagents in deionized H_2O for a final volume of 1 L. Adjust the pH to 7.8 with 10 M NaOH, and sterilize by autoclaving. Store indefinitely at room temperature.

HEPES	11.92 g
NaCl	51.43 g
KCl	0.75 g
NaHCO_3	2.10 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	2.46 g

3. To prepare 1× MBS, combine 100 mL of 10× MBS salt solution and 7 mL of 0.1 M CaCl_2 with 893 mL of autoclaved deionized H_2O . Add antibiotics. Store for up to 1–2 mo at 16°C–18°C (the same temperature used for oocyte incubation). Check before use.

The final reagent concentrations in 1× MBS are 0.7 mM CaCl_2 , 5 mM HEPES, 88 mM NaCl, 1 mM KCl, 2.5 mM NaHCO_3 , and 1 mM MgSO_4 .

Oocyte Culture Medium (OCM)

Reagent	Amount to add	Final concentration
Leibovitz's L-15 medium with L-glutamine	0.35 L	70%
Bovine serum albumin (fraction V)	0.2 g	0.4 mg/mL
Penicillin-streptomycin (100×/100×, P4333 Sigma-Aldrich)	5 mL	100 U/mL penicillin
		0.1 mg/mL streptomycin

Bring the volume to 0.5 L with deionized H_2O , and adjust the pH to 7.8 with NaOH. Filter to sterilize. Store at 4°C–8°C for up to 1–2 mo. Check before use.

Oocyte Reagent 2 (O-R2)

1. Prepare Stock Solution A by dissolving the following reagents in deionized H₂O for a final volume of 1 L. Store indefinitely at 4°C–8°C.

HEPES	11.915 g
NaCl	48.221 g
KCl	1.864 g
CaCl ₂ · 2H ₂ O	1.47 g
MgCl ₂ · 6H ₂ O	2.03 g
NaOH	1.520 g
2. Prepare Stock Solution B by dissolving 1.420 g of Na₂HPO₄ in deionized H₂O for a final volume of 1 L. Store indefinitely at 4°C–8°C.
3. Combine 100 mL of Stock Solution A, 100 mL of Stock Solution B, and 800 mL of deionized H₂O. Add antibiotics. Store for up to 1–2 mo at 16°C–18°C (the same temperature used for oocyte incubation). Check before use.

The pH should be 7.8. The final reagent concentrations are 5 mM HEPES, 82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 3.8 mM NaOH, and 1 mM Na₂HPO₄.

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